Control of Artifacts in Plasma Adenosine Determinations (43097)

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Abstract. **The literature concerning the role of adenosine (ADO) in physiology reveals no agreement about plasma ADO concentrations and suggests two main sources of error in these determinations: rapid ADO uptake by red blood cells or rapid ADO production from ADO nucleotides, which may be released by any cell lysis or platelet aggregation during plasma preparation. We therefore studied ADO concentrations in plasma from normal human forearm venous blood. ADO was determined by a high-performance liquid chromatographic procedure with a sensitivity of 3 n*M* (original plasma). Observed ADO concentrations ranged from 894 n*M* to 8.2 n*M* depending on the conditions of plasma preparation. In plasma prepared in plastic tubes from 4.5 ml of blood drawn into a plastic syringe containing 1.5 ml of an isotonic stopping solution (pH 7.4) containing heparin (60 units ml), dilazep (40 μM), EGTA (40 m*M*, EDTA (40 m*M*), *erythro*-9-(2-hydroxy-3-nonyl) adenine (40 μM), and α,β-methylene adenosine-5′-diphosphate (525 n*M*), the plasma ADO concentration was 13.3 ± 1.88 n*M* (SE) after correction for a simultaneous ADO recovery determination. The mean ADO recovery was 78% ± 3.39. The mean plasma ADO concentration found by this method of collection and preparation is lower then reported by others. Proper collection methods are required to avoid artifacts when determining plasma ADO concentrations.** [P.S.E.B.M. 1990, Vol 194]

Our interest in plasma adenosine (ADO) concentrations stems from concern about the sources of urinary ADO (1), for it is necessary to know how much can be filtered, reabsorbed, or secreted. There appears, however, to be no accepted value for plasma ADO concentrations. Reported mean values range from approximately 500 n*M* to 35 n*M* in human arm venous plasma (2–4), with a wide range of values in each study, which suggests that uncontrolled artifacts occur. Sources of error in these determinations include artifactual ADO formation from ADO nucleotides, released during platelet aggregation or from hemolysis (3, 5–7), as well as losses of ADO due to adenosine deaminase activity and cellular ADO uptake (6, 8), during plasma preparation. We therefore studied techniques of plasma preparation. The data indicate that artifacts can easily occur but that under conditions designed to inhibit both ADO formation and losses, plasma ADO concentrations do not exceed 20 n*M* in forearm venous blood from resting human subjects.

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Received March 1, 1989. [P.S.E.B.M. 1990, Vol 194]
Accepted March 13, 1990.

0037-9727/90/1944-0314$2.00/0
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Methods

Chemical Reagents. Adenosine (ADO), adenine (ADE), AMP, α,β-methylene adenosine-5′-diphosphate (AOPCP), EDTA, EGTA, nitrobenzylthioinosine (NBMPR), and trichloroacetic acid (TCA) were obtained from Sigma Chemical Co. (St. Louis). Dilazep (DZP) was provided by Hoffmann-La Roche (Basel). 2′-deoxycoformycin (DCF) (Pentostatin®) was a gift from the Warner-Lambert/Parke-Davis Co. (Ann Arbor, MI). *Erythro*-9-(2-hydroxyl-3-nonyl) adenine hydrochloride (EHNA) was a gift from the Wellcome Laboratories (Research Triangle Park, NC). Chloroacetaldehyde (4 *M*) was prepared as described previously (9). Organic solvents were of spectrophotometric grade. Diethylether of anesthesia grade and sodium heparin (USP) were used. Tritium-labeled adenosine ([2–8 ^{3}H] ADO), 30 Ci/m*M*, 1 mCi/ml, was obtained from Moravek Biochemicals Inc. (Brea, CA). Its purity was 98%. Scintillation fluid (Budget-Solve) was obtained from Research Products International (Mount Prospect, IL).

In studies described below, heparin was used to

inhibit clotting, either DCF or EHNA to inhibit adenosine deaminase (2, 5), a calcium chelator, EGTA, to inhibit platelet aggregation (7), a magnesium chelator, EDTA, which may also bind Zn^{2+}, to inhibit alkaline phosphatases (10), AOPCP to inhibit 5′-nucleotidase (11, 12), and either NBMPR or DZP to inhibit red blood cell ADO transport (8, 13). These agents were mixed in various combinations in "stopping solutions" added to whole blood at the time of drawing. These solutions were kept as close as possible to isotonic and at pH 7.4 to minimize hemolysis. Plastic syringes and tubes were used to avoid platelet aggregation.

Stopping Solutions. The stopping solutions contained the following concentrations of components (when present): heparin (all experiments), 60 units/ml; either DCF, 80 μM, or EHNA, 40 μM; either NBMPR, 36 μM, or DZP, 40 μM; AOPCP, 525 nM; EGTA plus EDTA, both 40 mM. A stock solution of EGTA and EDTA, both at 145 mM, mixed together, was first brought to pH 7.4. Its osmolality was 876 mOsm/kg. All stopping solutions were adjusted to 300 mOsm/kg with an appropriate amount of an NaCl solution (0.6 M). Final pH of the solutions was brought to pH 7.35 to 7.45, as determined just before use, with a few microliters of 6 N NaOH or 4.4 N H_3PO_4. When NBMPR was used, it was predried in the vessels from an ethanol solution.

Subjects, Blood Drawing and Handling. Two men and two women were used. They were 22 to 33 years of age and denied any type of drug intake and had fasted overnight. One volume of stopping solution was mixed with three volumes of whole blood. Blood was drawn from a forearm vein via a 19-gauge "butterfly" into plastic syringes prefilled with stopping solution. The butterfly was then disengaged and 1 ml of air was admitted to the syringe, followed by three inversions to mix contents. The blood-stopping solution mixtures were then transferred to plastic Eppendorf tubes and centrifuged at 15,000g for 1 min at room temperature to separate cells. The time elapsed between the start of blood drawing to the start of centrifugation ranged between 60 and 120 sec in initial experiments and from 60 to 90 sec in final experiments (No. 5–9). Cell-free supernatants, taken well above the cell layer, were transferred to 12- × 75-mm borosilicate glass tubes and treated with 25% TCA at a ratio of 0.25 ml of TCA to 1.0 ml of supernatant. The TCA-treated samples were chilled in ice for 15 min after vigorous mechanical mixing and centrifuged for 30 min at 4°C at 2000g. The supernatants were then extracted four times with equal volumes of water-saturated diethylether followed by heat treatment with buffered chloroacetaldehyde to prepare the fluorescent ethenoderivatives of ADO, ADE, and AMP (E-ADO, E-ADE, and E-AMP, respectively), as described previously (9), except that the chloracetaldehyde step was slightly modified. A mixture of 0.45 M sodium acetate (pH 5.0) and 0.36 M chloroacetaldehyde was added to the ether-extracted TCA supernatants at a ratio of 0.125 ml of the chloroacetaldehyde/acetate to 1.0 ml of supernatant. After a second set of ether extractions, the samples were ready for analysis.

Determination of E-ADO, E-ADE, and E-AMP. The samples prepared as described above were analyzed for E-ADO by high-performance liquid chromatography (HPLC). Initial studies used a method described previously (9). It was found necessary to improve the sensitivity of the method and to remove interfering substances by chromatographing the samples first on a different system, collecting fractions that were dried, then reconstituted and rechromatographed using a modification of the original HPLC method. All HPLC assays employed a Regis S5 (5 μm) ODS (C-18) column (20 cm × 4.6 mm inside diameter) preceded by a Brownlee RP-18 precolumn. The mobile phase flow rate was 1.0 ml/min with a 15-min linear gradient from the initial buffer (A) to the second buffer (B), and with a 7-min recycling time. Chart speed was 8 mm/min. The exciting wavelength for the fluorescence detector was 315 nm, emitting wavelength 415 nm, with 15-nm slits.

Four types of gradient elution systems were used to separate E-ADO, E-ADE, and E-AMP as follows:

Methods B1 and B2—Buffer A was 0.020 M sodium tetraborate ($Na_2B_4O_7 \cdot 10\ H_2O$) brought to pH 7.5 with 4.4 N H_3PO_4. Buffer B was 450 ml of A mixed with 200 ml of methanol. A 50-μl injection loop was used in B1 and a 200-μl loop in B2.

Method B3—This was like B1 and B2 but after preparing Buffer B, 2.5 ml of pure triethylamine was mixed with 550 ml of Buffer A, which was then degassed without refiltering. Buffer B contained no triethylamine. A 200-μl injection loop was used.

Method P1—Buffer A was 0.025 M potassium phosphate (pH 7.5). Buffer B was 450 ml of A mixed with 200 ml of methanol. A 50-μl injection loop was used.

Method P2—Buffer A was 0.007 M potassium phosphate (pH 6.9). Buffer B was 450 ml of A mixed with 200 ml of methanol. A 200-μl injection loop was used.

Methods B1 and B2 were used to determine E-ADO in initial experiments. Method B3 was used to determine E-ADO after a prior fraction-collection step with P2. P1 was used to determine E-AMP in preparations that had not been subjected to a prior fraction-collection step.

Method P2 was used when a second chromatographic step was necessary. A 2.0-ml plastic microcentrifuge tube held under the outflow tube of the flourescence detector collected the E-ADO fraction, which

was dried on a rotary evaporator under vacuum at room temperature. This was then reconstituted in 300 μl of water, with vigorous mixing and recentrifuging to bring the aqueous material to the bottom of the tube. Aliquots (200 μl) were assayed according to Method B3 (Experiments 2–9).

Standards and Blanks. Water solutions of ADO at concentrations expected to occur in the plasma, carried through all steps with the plasma preparations, served as internal standards. In Experiments 5–8 these were also prepared in stopping solutions. Reagent blanks were similar stopping solutions with no added ADO. External standards were water solutions of ADO, ADE, and AMP treated only with chloroacetaldehyde followed by a single set of ether extractions. In Experiment 9, [2-8 ^{3}H]ADO was added to the stopping solution to determine its recovery.

Scintillation Counting. Scintillation counting of tritium was done using a Beckman LS 6000TA counter with windows set at 0–400. Either 50-μl volumes taken from preparations or fractions collected during HPLC runs were used. These were placed in 7-ml vials which were then filled with scintillation fluid.

Calculations. Plasma ADO concentrations were calculated from net peak heights of unknowns and internal standards, as given previously (9). Plasma AMP concentration was calculated from external standards.

Recovery of ADO added to blood preparations was calculated from the observed difference in plasma concentrations between pairs of samples, with and without added ADO, divided by the theoretical concentration difference if recovery was 100%. The theoretical concentration difference was calculated as the amount of ADO added to the preparation divided by the volume of plasma to which it was added. The plasma volume was calculated from the volume of blood used and its hematocrit.

Recovery of E-ADO in the fraction-collection-reconstitution steps (Method P2 followed by B3) was calculated from standard solutions of E-ADO that had and had not been taken through this procedure.

The sensitivity of each HPLC method was calculated as the original plasma ADO concentration that would produce a net peak height of 2.5 mm in the final HPLC method used. When ADO recovery trials were done, the sensitivity calculation took this recovery into account.

Scintillation counts per minute were converted to disintegrations per minute from the efficiency determination for each vial. These efficiencies ranged from 47 to 54% in individual vials.

Details of Individual Experiments. Nine experiments are presented. Experiment 1 is one of four initial experiments which gave similar results, using HPLC Methods B1 or B2, and which were previously reported in abstract form (14).

Experiments 1–4 in the present report were done with blood from the same male subject. In Experiment 4, four blood samples were drawn at approximately 15-sec intervals into stopping solutions of differing composition. ADO recovery trials were done in Experiments 5–8. In these, two blood samples were drawn at approximately 15-sec intervals, first into stopping solution containing no added ADO, and second into stopping solution containing added ADO (66 n*M* in the stopping solution). ADO recovery was also studied in Experiment 9 using [2-8 ^{3}H-ADO] at 15.4 n*M* in the stopping solution (8.36×10^5 disintegrations per min/ml).

Plasma ADO concentration was determined by HPLC Method P2 followed by B3 in Experiments 2–9. Plasma AMP was also determined by HPLC Method P1 in Experiments 5–8.

Results

HPLC Methodology. Figure 1 illustrates HPLC tracings from the methods used and shows the separations of E-ADO, E-ADE, and E-AMP.

Linearity, Reproducibility, and Sensitivity of E-ADO Determinations. In all methods the net peak height of E-ADO standards was linearly related to injected concentration. In Method B3, the coefficient of variance was 5.3% at 17.3 n*M* injected concentration ($n = 5$). The sensitivity of Methods B1 and B2 was approximately 15 n*M*, while that of Method P2 followed by B3 was 3 n*M*.

Recovery of E-ADO in Methods P2 and B3. The mean recovery of E-ADO in the fraction-collection-reconstitution steps was 85.1% ± 2.57 (SE) of the theoretical recovery. Duplicate recovery determinations agreed closely in each trial. Since plasma ADO concentration calculations were based on internal standards, this recovery is taken into account.

Effects of Stopping Solution Composition on Observed Plasma ADO concentration. Table I shows the effect on observed plasma ADO concentration of the composition of the stopping solution. In the four initial experiments illustrated by Experiment 1, the plasma ADO concentration fell at or below the sensitivity of the HPLC method used (15 n*M*). In Experiments 2 and 3, which were similar to Experiment 1 except that DZP was used in place of NBMPR, low but measurable plasma ADO concentrations were found with method P2 followed by B3. Experiments 4a–4d reveal marked differences in observed ADO concentrations in plasma from the same blood dependent on the presence or absence of AOPCP or the combination of EDTA and EGTA. AOPCP alone reduced the ADO concentration markedly from that found in the absence of AOPCP and the chelators. The chelators were even more effective in reducing observed plasma ADO concentration, and in their presence there is no evidence of additive effect of AOPCP. The small difference in the results of

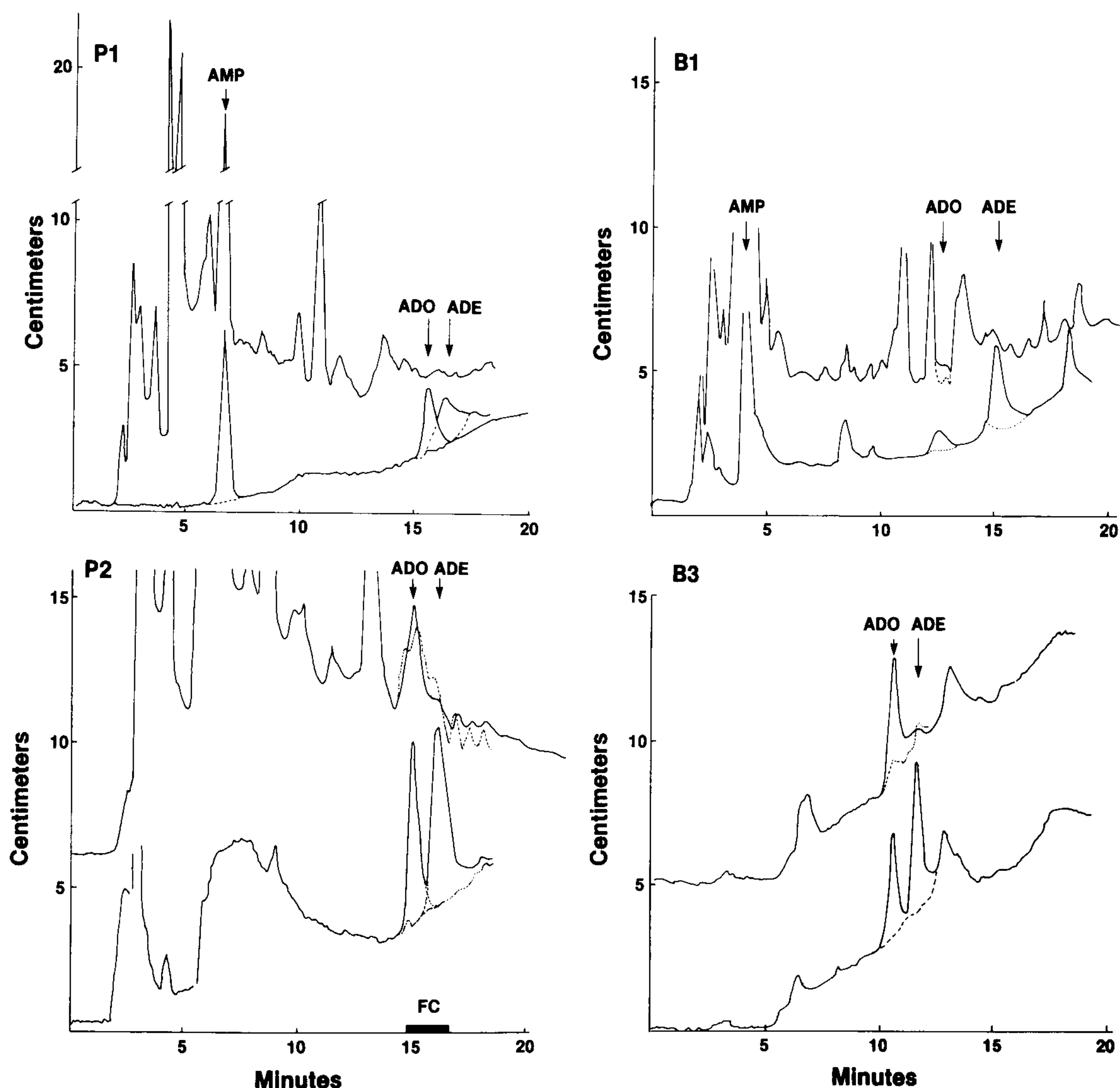

Figure 1. Representative HPLC tracings. In each panel the lower solid line shows standards and the upper solid line, plasma preparations. Dotted lines show portions of tracings from preparations from the same set which contained no added AMP, ADO, or ADE (see arrows), superimposed on those which did, but were otherwise essentially identical. Panel P1: AMP determination by Method P1. Injected concentration of E-AMP in standard, 44 n*M*; E-ADO, 34.5 n*M*; E-ADE, 34.5 n*M*. Panel P2: fraction-collection step with Method P2. Solid bar (FC) on horizontal axis shows the E-ADO collection period (1.8 min). Injected standard concentrations: E-ADO, 17.3 n*M*; E-ADE, 34.5 n*M*. Panel B1: Method B1. Injected internal standard concentrations: E-ADO, 30 n*M*; E-ADE, 57.8 n*M*. The E-AMP peak illustrates its separation from the others. Panel B3: Method B3. Injected internal E-ADO standard concentration (shown) was 10.5 n*M* as calculated from external standard data (not shown). This internal standard is the collected-reconstituted standard injected at 17.3 n*M* shown in Panel P2. The E-ADE peak (Panel B3) is from an external standard (17.3 n*M*).

Experiments 4c and 4d may not be significant, given an analytical sensitivity of 3 n*M*. Comparison of Experiments 4c and 4d with Experiments 2 and 3 suggests that slightly higher plasma ADO concentrations were found when the stopping solution contained EHNA in place of DCF, and DZP was present.

The results of Experiments 5–8 are combined in Table I, showing the mean observed plasma ADO concentration from a determination done on each of the four subjects, using stopping solution containing AOPCP, the chelators, EHNA, DZP and heparin. The mean concomitant ADO recovery value was 78% ± 3.39 (SE).

In Experiment 9, 87% of expected tritium disintegrations per minute was found in the plasma-stopping solution mixture just after the first centrifugation (before treatment with TCA or chloroacetaldehyde). HPLC data indicated that 98% of this material was in the ADO fractions. The total observed plasma ADO concentration was calculated to be 18.5 n*M*, of which 9.9 n*M* could be attributed to ADO added in the stopping solution. The difference (8.6 n*M*) corrected for the recovery of 87% gives 9.9 n*M* endogenous ADO in the original plasma.

Plasma AMP Concentrations. AMP determinations were done in Experiments 5–8 on both preparations from each experiment. The calculated mean value was 174 n*M* ± 35.3 (SE, $n = 8$).

Table I. Effects of Stopping Solution Composition on Observed Plasma ADO Concentration

Experiment	Stopping solution content[a]						ADO (n*M*)
	DCF	EHNA	NBMPR	DZP	EDTA + EGTA	AOPCP	
1	+	−	+	−	+	+	<15
2	+	−	−	+	+	+	10.3
3	+	−	−	+	+	+	7.5
4a	−	+	−	+	−	−	894
4b	−	+	−	+	−	+	314
4c	−	+	−	+	+	−	14.1
4d	−	+	−	+	+	+	19.9
5–8	−	+	−	+	+	+	10.2 ± 1.17[b] (mean; SE)

[a] All solutions contained heparin. +, present; −, absent.
[b] After correction for ADO recovery data in each of these four experiments, the mean (ADO) was 13.3 ± 1.88 (SE).

Discussion

The low plasma ADO concentrations from human forearm venous blood observed in our experiments are well below levels reported by others (2–4). Our experiments demonstrate that differing methods of blood collection result in artifactually high plasma ADO concentrations. In the absence of EGTA, EDTA, and AOPCP but with DZP, EHNA, and heparin present, the observed plasma ADO concentration was 894 n*M*. This was reduced in plasma from the same blood to 314 n*M* by the further addition of AOPCP alone. Both of these values are at or above levels reported by others (2, 3). The reduction in ADO concentration caused by AOPCP indicates that 5′-nucleotidase activity is responsible for some of the ADO observed in the absence of AOPCP and the chelators (11, 12). When EGTA and EDTA were both present, with or without AOPCP, no observed plasma ADO concentration exceeded 20 n*M* even after correction for ADO recovery data in Experiments 5–9.

Both types of recovery trial, using either native ADO or [2–8 ^{3}H]ADO, indicated recovery of 75 to 87% of added ADO. Experiment 9 indicates that this loss occurred prior to the end of the first centrifugation. It may have occurred during blood drawing before mixing of blood and stopping solution was complete.

Significant concentrations of AMP were found in the preparations in Experiments 5–8. It is not known whether such AMP concentrations exist in normal, circulating blood plasma but it is known that the arabinosyl analog of AMP is very rapidly converted to arabinosyladenine after injection in man (9). The AMP observed in our preparations could arise from ADO nucleotides released during platelet aggregation (7, 15) or from even minor cell lysis, in view of the very high concentrations of ADO nucleotides in human red cells and leukocytes (16). AMP formed by these mechanisms could be a main source of artifactual ADO formation if 5′-nucleotidase and phosphatases are not inhibited. We speculate on the basis of work of others (5, 7, 10, 12, 17) that EGTA and EDTA, combined in the concentrations used, reduce the concentrations of Ca^{2+}, Mg^{2+}, and Zn^{2+} enough to suppress platelet aggregation and also phosphatase and 5′-nucleotidase activities. Although total suppression of these activities by the chelators has not been demonstrated by these studies, the findings indicate that ADO was formed rapidly and in large amounts when the chelators were not present.

The losses of ADO noted in Experiments 5–9 could be due to cellular uptake of ADO, or to the action of adenosine deaminase. The concentration of DZP used in these experiments has been shown to reduce red blood cell ADO uptake to that due to simple diffusion (8). At low plasma ADO concentrations, however, if passive diffusion remains uninhibited, ADO might diffuse from cells to plasma if the concentration gradient permitted. ADO concentrations in human red blood cells have been given as 200 n*M* (18), which is far above the plasma ADO concentrations observed by us when EGTA and EDTA were both present in the stopping solution. With respect to adenosine deaminase activity, the concentration of EHNA used would be expected to inhibit this almost completely (19). EHNA is probably preferable to DCF as an adenosine deaminase inhibitor in studies of plasma ADO concentrations because the onset of its effect on the enzyme is more rapid (19).

Some recent reports also have suggested that plasma ADO concentrations are lower than previously thought. One group reported a mean value of 35 n*M* in human blood (4) and another group reported a mean value of 40 n*M* in swine blood (5). In both of these studies, EDTA alone (4m*M*) was present in the stopping solution, dipyridamole was used, and EHNA was present. DZP has been shown to be preferable to both dipyridamole and NBMPR as an ADO transport inhibitor (8). We believe that our lower observed plasma ADO concentrations are largely due to the use of EDTA at higher concentrations than used by others, and perhaps also to the use of EGTA. The optimal concentrations of these agents and of AOPCP in the stopping solutions remain to be determined more fully.

Proper collection methods are required to avoid artifacts when determining plasma ADO concentrations. We believe that the plasma ADO concentration in normal human forearm venous blood is less than 20 n*M*.

This work was supported by Grant HL 38688 from the National Heart, Lung, and Blood Institute.

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